PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: METHODS AND MATERIALS FOR DETERMINING RELATIVE ABUNDANCE OF MICROORGANISMS IN MIXED POPULATIONS

(57) Abstract

    The present invention relates to a method for determining the relative abundance of individual species or lower phylogenetic subgroups of microorganims in a mixed population of several microorganisms comprising the steps of: 1) providing a set of labelled in situ hybridization cluster oligonucleotide probes; 2) hybridization of said probes with a sample of the mixed population, and 3) quantitative analysis of the number of labelled microorganisms. Further it relates to a method for analyzing dynamics in relative abundance of individual microorganisms in a mixed population. Further it relates to a set of probes which are cluster specific and which are provided with at least one label for use in method of the invention and to a kit of parts for determining the relative abundance of individual species of microorganisms in a mixed population of several microorganisms comprising such probes together with suitable materials for pretreating the sample and/or suitable materials for carrying out hybridization and/or suitable materials for analysis of the result of the hybridization.

Title: Methods and materials for determining relative
abundance of microorganisms in mixed populations.

The invention relates to methods and materials for
determining the relative abundance of individual
microorganisms in mixed populations of microorganisms, in
particular to such methods for determining relative abundance
5   of individual microorganism species in the indigenous gut
flora, especially of humans.
     Such methods have not been available until the present
invention. What has been possible until the present invention
is determination of the presence of individual microorganism
10   species in a mixed population such as the intestinal flora.
     Traditional methods to determine the composition of the
intestinal flora require culturing of the microorganisms in
the population on both selective and non-selective media.
These methods are laborious and time consuming, which in
15   itself makes it hardly possible to do anything beyond
identifying the microorganisms present in the mixed
population. Furthermore these methods are prone to
methodological and statistical error. Another barrier for
determination of abundance of one microorganism vis à vis
20   others (relative abundance) is that culturing includes
multiplication of the microorganisms which of course do not
divide at the same rate. Therefore determination of relative
abundance is practically not possible using these methods, let
alone that the dynamics of such a population through time
25   could be followed.
     The composition and activity of the indigenous gut flora
is of paramount importance to human immunology, nutrition,
pathogenesis and hence to health of individuals (Van der Waaij
et al.,1971). Colonization resistance, or bacterial antagonism
30   of the indigenous intestinal microflora for instance
represents a first line of defence against the establishment
of pathogenic microorganisms in the intestinal tract (Van der
Waaij et al.,1971;1989).

The human (mammalian) intestinal tract is not the only mixed population of microorganisms for which measuring the relative abundance of individual species or other subgroups of microorganisms is useful or important.

5   In industry, specifically in the food sector and fermentation industry, monitoring of the bacteria- or yeast-dependent fermentation process is essential for quality assurance both with respect to hygiene and product stability. Environmentally, monitoring of complex mixed bacterial

10   populations is elemental in predicting the effectiveness of bioremediation in soil-, gas- and waste water-treatment. Environmental microbiologists are in need for tools that explain nutrient regeneration potentials in terms of biomass of individual metabolic clusters of bacteria.

15   Although much is known of the physiology of most bacterial species individually and of metabolic activities of mixed bacterial consortia as a whole, factors that determine their abundance and those causing subtle as well as dramatic changes in population composition have remained largely

20   unknown due to the technical limitations of the classical cultivation and identification techniques. Therefore, rapid monitoring techniques that can unravel the population compositions of complex mixed bacterial consortia are of great scientific importance.

25   The invention thus provides a rapid and reliable method for determining the relative abundance of individual species or lower phylogenetic groups of microorganims in a mixed population of several microorganisms comprising the steps of:

       1) providing a set of labelled in situ hybridization

30   cluster oligonucleotide probes;

       2) hybridization of said probes with a sample of the mixed population,and

       3) quantitative analysis of the number of labelled microorganisms.

35   In situ hybridization in itself is a well known technique, as can be read from EP-A-0 497 464, which relates to in situ hybridization techniques using fixed, permeabilized

but not mounted cells. The method is used to determine the
presence of single microorganisms. So far in situ
hybridization has however not been applied to determine the
relative abundance of lower phylogenetic subclasses, let alone
5 following the dynamics of a mixed population of
microorganisms. Labelled probes are an essential part in in
situ hybridization. The present invention provides a set of
cluster probes which have been chosen in an unexpected novel
manner, i.e. not based on the normal taxonomic principles. A
10 set of cluster probes for the purposes of this invention is
defined as a set of oligonucleotides capable of identifying
microorganisms from a cluster, such as a species, a genus or
another lower phylogenetic subgroup. Preferably these probes
are all based on a comparable target nucleic acid of the
15 different microorganism in the mixed population, in this case
for example the 16S rRNA.

    Cluster-probes have received most attention in aquatic
and soil microbiology. The limitations of using culture-
dependent methods for studying the diversity and ecology of
20 natural microbial communities are well known in these fields
since most (>99%) of the bacteria present in these habitats
are non-culturable and of unknown taxonomic identity. As a
consequence, the use of more direct molecular
characterizations has become more common in this field. A
25 recent review (Amann et al., 1995) summarizes many of the
lower group level/genus level proves developed thus far.
Invariably, these probes find their application in the
environmental fields of waste water and sediment microbiology.
Upper group level probes (such as Proteobacterial subclass
30 probes)and domain level probes (used to distinguish between
archaea, bacteria and eucarya) have limited descriptive value
since they "cover" too many different evolutionary lines of
descent. The application of species-level probes in the
analysis of the community structure of complex mixed bacterial
35 assemblages will probably make the method equally time
consuming as traditional cultivation techniques.

These types of cluster probes are therefore not suitable for the present invention. In the field of regular use of cluster probes nobody has contemplated using lower phylogenetic cluster probes, let alone that anyone would have
5   applied such probes to the field of determining the relative abundance of microorganisms in human intestinal flora.

These 16S rRNA probes are a preferred embodiment of the present invention.

With increasing frequency, 16S-rRNA targeted
10   hybridisation probes in combination with PCR-amplification of target DNA, are applied for the detection of specific bacterial taxa in mixed bacterial populations. Currently, by using PCR techniques the qualitative presence of a single cell against a background of 10^9 non-target cells can be confirmed.
15   As such the technique is extremely sensitive. However, the technique gives little or no quantitative information on absolute cell numbers. Fluorescence in situ hybridisation with a set of phylogenetic cluster probes is superior to PCR with respect to enumeration of individual cells that belong to a
20   certain bacterial group. This technique is also superior to the DGGE-technique for mixed population analysis of which PCR forms an integrated part.

Approximately 100 bacterial species out of ± 400 dominate the culturable (and thus identified) population. The 16S rRNA
25   sequences of most of these bacteria are currently known. There is more than sufficient sequence data to design specific probes for predominant groups of faecal bacteria. We report the development of a set of 15 16S rRNA targeted oligonucleotide probes with which the predominant bacterial
30   groups of the human intestinal micro flora can be enumerated in situ.

The probes are preferably labelled with fluorescent materials. This allows for microscopic analysis combined with preferably automated image analysis.
35   For the detection of fluorescent oligonucleotide probes hybridized to bacteria on microscopic slides, photography can be applied. However, quantification by this method is hampered

by the absence of an objective threshold criteria for the
discrimination between hybridized and nonhybridized cells.
Therefore, for objective evaluation of probe specificity, an
image analysis system is employed which allows fluorometrical
5   measurement of individual cells. All details of the hardware
and software of this system have been published elsewhere (see
refs. 32-34 in Bifidobacterium-paper of Langendijk et al.,
aug. 1995).

Also preferred is an embodiment where probes identifying
10   a certain species are provided with a different label than
other probes identifying other species. This reduces the
number of assays and/or samples to be taken.

The invention also provides probes and kits for carrying
out the methods according to the invention.

15   The kits will usually comprise probes and other reagents
for _in situ_ hybridization, as well as other reagents for
pretreating samples and/or post treating samples.

Applications of the invention
20   In such a kit, the probes may be labelled with
fluorescein-5-isothiocyanate (FITC) at the 5'-end. Other
labels may be attached to either 3'- or 5'-end of these
probes, to both ends or internally.

25   Among these labels can be
1)   direct fluorescent labels:
-    FLUOS (5(6)-carboxyfluorescein-N-hydroxysuccimide-ester)
-    TRITC (tetramethylrhodamine-5-isothiocyanate)
-    AMCA (7-amino-4-methylcoumarin-3-acetic acid)
30   -   Texas red
-    Indocarbocyanine dyes such as CY3, CY5 or CY7
-    Any other fluorescent label.

2)   indirect labels:
35   -   Enzymes such as alkaline phosphatase (AP) or horseradish
peroxidase (HRP) either attached directly or via a C6 thiol
linker and used in combination with chemiluminescent

6

substrates like AMPPD (3-('-spiroadmantane)-4-methoxy-4-(3'-phosphoryloxy)-phenyl-1,2-dioxethane) or fluorescence-generating substrates.

-       Digoxigenin
5   in combination with anti-DIG antibodies labelled with
        - gold particles
        - fluorescent labels such as carboxyfluorescein or carboxyrhodamine 101
        - enzymes such as alkaline phosphatase (AP) or
10 horseradish peroxidase (HRP) in combination with chemiluminescent substrates like 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)-phenyl-1,2-dioxethane (AMPPD) or fluorescence-generating substrates.

-       Biotin
15   in combination with avidin or streptavidin and labelled as the anti-DIG antibodies

-       Dinitrophenyl (DNP) as hapten
        in combination with appropriate antibodies and labelled as the anti-DIG antibodies
20 -   or any indirect label.

Further objects of the invention are:
        To study the effect of novel foods (prebiotics), probiotics and functional foods on the intestinal microflora
25 of both man and animal;
-       To study the effect of antibiotics on the intestinal microflora of both man and animal;
-       To monitor the development of mixed microbial populations that are involved in industrial fermentation processes (cheese
30 and other (dairy) fermentation products, beer, wine etc);
-       To rapidly detect microbial contamination in product or production tools from raw material to end product (hygiene control);
-       To identify and quantify the presence and activity of
35 micro-organisms in waste water treatment systems;
-       To identify and quantify the presence and activity of micro-organisms in bioremediation processes;

- To identify and quantify the presence and activity of micro-organisms in marine systems;

- To determine the microbial status of (polluted) soils, waters, gasses etc;

- To determine the status of potable water, swimming water and surface water with respect to pathogenic bacteria and human health;

- To study the effect of registered and novel medicines on the intestinal microflora of both man and animal;

- To perform diagnostics/rapid detection of pathogenic infections in man and animal including livestock;

- To detect bacterial resistance to antibiotics;

- To study the effect of homeopathic preparations and reform products on the intestinal microflora of both man and animal;

- To detect specific tooth-decay generating oral streptococci for the toothpaste industry;

- To detect pathogens or consortia of microorganisms that are either detrimental or beneficial to (crop) development, yield or quality during production, harvest, storage and transport.

The invention is further illustrated in the following detailed description.

Detailed description of the invention.

Development of the set of gut flora cluster-probes

In order to elucidate the population composition of complex mixed bacterial populations, aerobic and anaerobic cultivation techniques on solidified media are routinely employed. Standard identification protocols or commercially available test systems are subsequently used to identify the isolated strains. Although it is relatively easy to obtain a total viable count, counting of individually identifiable bacterial species is laborious and time consuming. Viable

counts of the various aerobic and facultatively anaerobic
species can be obtained by using selective media. However,
such media are not available for most strictly anaerobic
species of the gut flora. Since some 30-40 species account for

5   99% of the human gut flora, several hundreds of isolates from
each individual sample have to be identified in order to
provide reliable population statistics. Furthermore,
fermentation end-product analysis in isolated pure cultures is
often essential for reliable identification. As a result,

10 studies on population dynamics of the intestinal flora are
often limited in the number of subjects or the number of
species investigated and, as a consequence, the observed
changes in composition are often not statistically
significant. The recent increase in the production of designer

15 foods (functional foods, pre- and probiotics) has lead to a
demand for tools to study the effect of these products on the
human intestinal microflora. We have developed such a tool by
combining several innovative microbiological techniques such
as fluorescence in situ hybridization and image cytometry.

20   Specifically, we have developed 16S rRNA targeted
oligonucleotide probes for the enumeration of various
phylogenetic groups of intestinal bacteria. These probes are
used in fluorescence in situ hybridization studies on
population dynamics. We have developed these probes after

25 demonstrating the presence of fifteen major phylogenetic
subpopulations within the intestinal microflora (Fig.1).

Image analysis and FISH

30   Whole-cell in situ hybridization with 16S rRNA targeted,
fluorescently labelled, synthetic oligonucleotides facilitates
rapid determination of the taxonomic position of individual,
even unculturable cells and allows accurate description of
bacterial populations on a quantitative basis via automated

35 microscopic image analysis. With the combination of these two
techniques, it is possible to measure the fluorescence of
specific cell-types quantitatively. The use of image analysis

enables quantification of even very low fluorescent signals
and ensures objective (researcher-independent) evaluation.
hybridization results.

Automated recognition and enumeration of hybridized cells is
5   facilitated by the use of image analysis software developed in
our department (Meijer et al., 1991; Wilkinson, 1994;
Wilkinson et al., 1993, 1994).

During an extensive study, using cultural methods of
enumeration, some 200 species of bacteria have been isolated
10   from human faecal samples (Finegold et al., 1974).
Approximately 30 of these are numerically important and
represent 10 distinct genera. Genus specific probes could thus
prove appropriate in describing community structures at large.
However, the natural classification system for bacteria, based
15   on phenetic characteristics, is known no to comply fully with
genetically based methods; not all taxonomic genera are
monophyletic on the basis of their 16S rRNA sequence,
Eubacterium spp., for example, are clustered with several
Clostridium and Peptostreptoccus spp., and peptostreptococel
20   themselves are spread over several distinct phylogenetic
clusters (Fig. 1), within these phylogenetic clusters,
however, conserved regions can be identified and 16S rRNA
probes designed for such regions can be used to identify the
majority of the population to a well defined taxonomic level.
25   Only a limited number of probes is thus required for the
enumeration of the various phylogenetic groups of the gut
flora (Fig.1).

Although many copies of the ribosome should ensure
sufficient signal, reality is often far from ideal. The
30   accessibility of the target region, the number and kind of
fluorochromes attached to the probe, the hybridization
temperature, the metabolic status of the cells and the level
of autofluorescence of non-target cells are important factors
in obtaining sufficient specific fluorescent signal. Optical
35   aid in the form of CCD-camera's is often required for small
and slowly growing cells with little rRNA. Image analysis can
be very helpful in discriminating between non-fading

autofluorescence and rapidly fading fluorochrome fluorescence.
Using such fluorescence lifetime imaging techniques, only a
few positively hybridized cells can be distinguished among
several thousand non-target cells in a microscope field
5   (manuscript in preparation). This is necessary to enumerate
those bacterial clusters that form a minority of the
population, (e.g. Streptococci).

MATERIALS AND METHODS

10

Maintenance of cultures
     Bacterial cultures were maintained in anoxic Chopped Meat
Carbohydrate medium (CMC) (Holdeman et al., 1977) at room
temperature with bi-monthly transfers, and in anoxic skimmed
15   milk at -20°C. Prior to hybridization, fresh cultures were
grown in anoxic Peptone/Yeast extract/Glucose medium (PYG)
(Holdeman et al., 1977) at 37°C.

Development of probes

20

     The 16S rRNA sequence of 113 species of faecal bacteria
was retrieved from the RDP database version of 18 June 1994
(Larsen et al., 1993). Together, these species represented
most bacteria known to occur in human faeces (Drasar and
25   Barrow, 1985). A Kimura 2 parameter distance analysis of the
aligned sequences of these bacteria was perfomed with the
Molecular Evolutionary Genetics Analysis (MEGA) program of
Kumar et al. (1993), version 1.02. The obtained distance tree
(Neighbour Joining) of the various species in phylogenetic
30   clusters was compared to the phylogenetic grouping of the RDP
database (Larsen et al., 1993). No significant cluster
differences were found. Fifteen clusters were thus identified
(Fig. 1). Unique sequences for each of the separate clusters
were identified and an estimate was obtained of the number of
35   cells in an average faecal sample that would hybridize with a
probe for that cluster. Probe specificity was considered
useful if the positively hybridizing part of the population

was between 1% and 25%. In the case that a single species was
expected to consist a major part of the total gut flora
population, a species-specific probe was developed for that
species.

5

Testing of probe specificity

Specificity of the probes was tested against the complete
RDP database using the Check Probe command (Larsen et al.,
10   1993) and was considered sufficient if a minimum of two
mismatches with bacteria from all of the phylogenetic clusters
of faecal bacteria was confirmed. Furthermore, a reference
collection of 86 bacteria (table 1), representing a subset of
the 113 species included in the target identification
15   procedure (Fig. 1), was cultered and whole cell _in situ_
hybridizations with these 86 species were performed. To this
end, exponentially growing cells were fixed with 3% (v/v)
formaldehyde. After 10 min storage at 4°C, 10 µl of fixed
cell-suspension was applied to a glass slide that was pre-
20   treated with Vectabond™ (Vector Laboratories, Burlingame, CA)
to ensure proper adhesion of the cells. After drying for 20
min. at 45°C, the dried cell smears were dehydrated in a
graded ethanol series (50%, 75% and 98% ethanol, v/v; 2 min.).
Twelve µl of 0.2 µm pore-size filtered hybridization buffer
25   was added to the cell smears. The hybridization buffer
consisted of 0.9 M NaCl, 20 mM Tris-HCl (pH 7.5) and 0.1%
(w/v) sodium dodecyl sulphate. After addition of
8 ng, µl^{-1} FITC-labelled probe, the smears were covered with a
coverslip. The slides were incubated in a buffer-saturated
30   hybridization chamber at 45°C for 15-20 h. After
hybridization, the slides were washed for 15 min. in 50 ml
hybridization buffer at 45°C, air-dried and the cell smears
were embedded in mounting fluid consisting of a 1 : 1 mixture
of glycerol and PBS (8g 'l^{-1} NaCl, 0.2 g 'l^{-1} KCL, 1.44 g 'l^{-1}
35   Na$_2$HPO$_4$, 0.24 g 'l^{-1} KH$_2$PO$_4$), supplemented with 2.5% (w/v) NaI.
The slides were evaluated by using an Orthoplan™
epifluorescence microscope (Leitz, Germany) equipped with a Hg

arc lamp (HBO 100W, Osram, Germany), a 50x PL fluotar
objective (Leitz), an I2/3 (blue excitation) filter block and
a cooled CCD video camera (Loral, Fairchild CCD 5000/L,
Sunnyvale, CA). An exposure extension system for this camera

5   was described previously (Wilkinson et al., 1993). The image
analysis software used was the Groningen Reduction of Image
Data (GRID) system (Meijer et al., 1991; Wilkinson et al.,
1994). The fluorescence measurements were performed using the
immunofluorescence package described previously (Jansen et

10   al., 1993). Surface fluorescence signals were calibrated by
using a non-fading uranyl glass reference (16). A phase-
contrast and fluorescence image of each field of view were
obtained. The shape of each bacterium was determined from the
phase-contrast image automatically. The fluorescence of each

15   object was determined from the corresponding area in the
fluorescence image. This procedure avoids exclusion of non-
fluorescent objects. The fluorescent images were recorded
with a camera exposure-time of 12 sec. Per microscopic slide,
250-500 objects were measured. For each slide, the negative

20   control (autofluorescence) was determined and the 95th
percentile of its fluorescence distribution served as a
threshold. The percentage of positively-hybridized objects,
with fluorescence above the threshold, per total number of
objects detected under phase-contrast illumination was dubbed

25   the hybridization percentage. This percentage was determined
to evaluate the performance of the probes and was done on pure
cultures.

Whole cell in situ hybridisation and enumeration of
30   phylogenetic clusters in faecal samples
     For quantification of phylogenetic clusters and total
anaerobes in faeces, stool specimens from healthy human
volunteers were collected and processed as follows. One gram
of freshly defecated and homogenized faeces was suspended in

35   9 ml of 0.2 μm pore-size filtered PBS. This suspension was
diluted 10 x in filtered PBS and thoroughly mixed. After
removal of debris (350 x g; 1 min) the supernatant was

collected and fixed overnight at 4°C with 4% (w/v) fresh
paraformaldehyde solution. Cells from 1 ml of fixed cell-
suspension were washed twice (8,000 x g; 5 min) in 1 ml of
filtered PBS and resuspended in 1 ml of a mixture of PBS and

5   ethanol (1:1). After 1 h of storage at -20°C, 5 µl of cell
suspension was added to 50 µl pre-warmed (50°C) hybridization
buffer and 5 µl of FITC-labelled probe was added. Cells were
hybridized for 16 h at 50°C. After resuspension in 10 ml
hybridization buffer, cells were filtered on a 0.2 µm pore-size

10 Isopore™ polycarbonate membrane filter (Millipore Corporation,
U.S.A.) and washed twice with 10 ml of warm (50°C)
hybridization buffer. Filters were mounted on microscope
slides with Vectashield™ and hybridized cells were counted
visually using an Olympus BH2 microscope with a

15 DPlanApo100UVPL objective (100x, N.A. 1.30), an HBO100 high
pressure mercury vapour lamp, and an IB blue light excitation
block with an extra EY455 excitation filter. The total number
of bacteria present in faeces was determined by the method of
Porter and Feig (1980), using 0.5 µg . ml^{-1} (w/v)

20 4',6-diamidino-2-phenylindole (DAPI) as a DNA stain under
illumination with a UV excitation filter block. All
microscopic counts were determined in duplicate, with a
minimum of 300 cells counted per assay.

25 Special pre-treatment procedure for the in situ hybridisation
of lactobacillus spp.
        Most bacteria exhibit high levels of fluorescence when
hybridised with fluorescent probes (Langendijk et al., in
press). However, members of the genus Lactobacillus are

30 difficullt to hybridize. Pure cultures of this genus exhibit a
very low level of fluorescence and, in general, less than 50%
of the cells exhibits fluorescence above background values
(Langendijk et al., in press). Such results have also been
reported for Gram-positive cocci such as lactococci,

35 streptococci and enterococci (Beimfohr et al., 1993,
Salama et al., 1991). That the poor permeability of these
cells is not solely due to their Gram-positive nature is

demonstrated by the fact that both clostridia and
bifidobacteria exhibit high fluorescence signals with 16S
probes when using standard hybridization conditions
(Langendijk et al., in press).

5   We have developed a protocol for whole cell _in situ_
hybridization of lactobacilli. In this protocol, standard
whole-cell fluorescence _in situ_ hybridization (FISH) protocols
are preceded by a lysozyme/lipase treatment as follows.

10 Determination of specificity of the Lab probe:
       Cell of 1 ml of mid-log cultures of 10 _Lactobacillus_
strains and a total of 26 reference stains (see tables 2 and
3), were washed in PBS and resuspended in 1 ml of pre-lysis
buffer. The PBS consisted of 8 g.l^{-1} NaCl, 0,2 g.l^{-1} KCl,
15 1.44 g.l^{-1} Na$_2$HPO$_4$ and 0.24 g.l^{-1} KH$_2$PO$_4$. The pre-lysis buffer
consisted of 50 mM Tris (pH 7.5); 10 mM EDTA; 0.585 M sucrose;
5 mM CaCl$_2$; 0.3 mg.ml^{-1} sodium taurocholate; 5 mg.ml^{-1} lysozyme
and 0.031 mg.ml^{-1} pancreatic lipase. Cells were incubated for 1
h at 37°C, washed in PBS and fixed in ethanol-PBS (1:1) for
20 1 h. Ten microlitre of this suspension was dried on a glass
slide and heat-fixed. The cells smears were dehydrated in a
graded ethanol series (70% and 96%, 3 min each). Ten μl of
hybridisation buffer and 1 μl of FITC-labelled probe (50.μl^{-1})
was added to the smears. The bybridization buffer consisted of
25 0.9 M NaCl, 20 mM Tris-HCl (pH 7.5) and 0.1% (w/v) sodium
dodecyl sulphate. The smears were covered with a coverslip and
the slides were incubated at 50°C for 16 h in a buffer-
saturated hybridisation chamber. Subsequently, the slides were
washed for 30 min in 50 ml hybridisation buffer at 50°C, air-
30 dried and mounted in Vectashield (Vector Laboratories, Inc.
Burlingame, CA). This treatment resulted in hybridisation
percentages of over 90%.

TABLE 1: Bacterial species included in the determination of the specificity of the various probes. All 15 probes (except for the Lab probe [see below]) are being tested against all of the bacteria indicated. Positive hybridization results (obtained of anticipated) are marked by XXX.

PROBE SPECIES	REC	CYL	PEP	RUM	LIT	ANA	FUS	HIS	LAB	STR	FRA	DIS	ATO	LEP	BIF
Atopobium minutum													XXX		
Bacteroides distasonis												XXX			
Bacteroides eggerthii											XXX				
Bacteroides fragilis											XXX				
Bacteroides thetaiotaomicron											XXX				
Bacteroides uniformis											XXX				
Bacteroides vulgatus											XXX				
Bifidobacterium adolescencis															XXX
Bifidobacterium angulatum															XXX
Bifidobacterium animalis															
Bifidobacterium asteroides															XXX
Bifidobacterium breve															XXX
Bifidobacterium bifidum															XXX
Bifidobacterium boum															XXX
Bifidobacterium cornutum															XXX
Bifidobacterium densium															XXX
Bifidobacterium globosum															XXX
Bifidobacterium infantis															XXX
Bifidobacterium longum															XXX
Bifidobacterium magnum															XXX
Bifidobacterium pseudolongum															XXX
Bifidobacterium subtile															XXX
Bifidobacterium suis															XXX
Clostridium aminovalericum XXX															
Clostridium barast								XXX							
Clostridium beijerinckii								XXX							
Clostridium bifermensans MLS					XXX										
Clostridium butyricum								XXX							
Clostridium carnis								XXX							

SPECIES PROBE	REC	CYL	PEP	RUM	LIT	ANA	FUS	HIS	LAB	STR	FRA	DIS	ATO	LEP	BIF
Clostridium clostridiiforme	XXX														
Clostridium cochlearium		XXX													
Clostridium indolis															
Clostridium innocuum		XXX													
Clostridium perfringens								XXX							
Clostridium paraputrificum								XXX							
Clostridium putrificum								XXX							
Clostridium scatalogenes								XXX							
Clostridium splanchnicus															
Clostridium sporosphaeroides														XXX	
Enterococcus faecalis									XXX						
Enterococcus faecium															
Eubacterium aerofaciens															
Eubacterium budayi								XXX							
Eubacterium contortum	XXX														
Eubacterium cilindroides		XXX													
Eubacterium formicigenerans	XXX														
Eubacterium lensum													XXX		
Eubacterium moniliforme								XXX							
Eubacterium nitrinogenes								XXX							
Eubacterium rectale	XXX														
Eubacterium ruminantium															
Eubacterium siraeum														XXX	
Eubacterium tenue					XXX										
Eubacterium tortmosum		XXX													
Eubacterium ventriosum	XXX														
Fusobacterium mortiferum							XXX								
Fusobacterium necrophorum							XXX								
Fusobacterium vanium							XXX								
Lactobacillus acidophilus									XXX						
Lactobacillus brevis NZT01									XXX						
Lactobacillus casei subsp.casei									XXX						
Lactobacillus delbrueckii subsp.lactis									XXX						
Lactobacillus fermentum									XXX						

SPECIES	PROBE	REC	CYL	PEP	RUM	LIT	ANA	FUS	HIS	LAB	STR	FRA	DIS	ATO	LEP	BIF
Lactobacillus helveticus HP																
Lactobacillus plantarum										XXX						
Lactobacillus salivarius subsp.salicinius										XXX						
Lactococcus lactis subsp.lactis P15											XXX					
Leuconostoc lactis C17										XXX						
Peptococcus asaccharotysicus																
Peptostreptococcus anaerobius							XXX									
Peptostreptococcus micros				XXX												
Peptostreptococcus tetradius				XXX												
Propionibacterium acnes																
Ruminobacter amylophilus																
Ruminococcus hansenii		XXX			XXX											
Ruminococcus productus					XXX											
Streptococcus bovis											XXX					
Streptococcus bovis											XXX					
Streptococcus equinus											XXX					
Streptococcus intermedius											XXX					
Streptococcus mutans (DSM)											XXX					
Streptococcus mutans (SLL)											XXX					
Streptococcus salivarius subsp.??											XXX					
Streptococcus sanguis											XXX					

SUBSTITUTE SHEET (RULE 26)

Table 2

Reference strains used for specificity tests of the Lab probe

Name:	Origin
Bacteroides distasonis	MMB 3335
5 *Bacteroides fragilis*	MMB 3407
Bacteroides thetaiotaomicron	RPHL 2
Bifidobacterium adolescentis	NIZO B659
Bifidobacterium infantis	ATCC 15697
Bifidobacterium longum	MMB 3277
10 *Clostridium beijerinckii*	MMB 3318
Clostridium bifermentans	NIZO B529
Clostridium carnis	DSM 1293
Enterococcus faecalis	MMB 2405
Enterococcus faecium	SLL 4
15 *Eubacterium aerofaciens*	DSM 3979
Eubacterium cylindroides	MMB 3291
Eubacterium lentum	ATCC 25559
Eubacterium rectale	ATCC 33656
Fusobacterium mortiferum	MMB 3040
20 *Fusobacterium necrophorum*	MMB HB130
Lactococcus lactis subsp.*lactis* P15	NIZO B1
Leuconostoc lactis C17	NIZO B630
Peptococcus asaccharolyticus	MMB 3351
Peptostreptococcus anaerobius	MMB 2828
25 *Peptostreptococcus micros*	DSM 20468
Ruminococcus productus	DSM 2950
Streptococcus bovis	SLG 1
Streptococcus equinus	SLL 5
Streptococcus intermedius	DSM 20573

<u>Table 3</u>

Lactobacillus strains used for specificity tests of the Lab
probe

Name:	**Origin**
5	*Lactobacillus acidophilus*	DSM 20077
Lactobacillus brevis NZT01	NIZO B306
Lactobacillus casei subsp.*casei*	MMB 3225
Lactobacillus delbrueckii subsp.*lactis*	NIZO B193 (ATCC 800)
Lactobacillus fermentum	MMB 3289
10	*Lactobacillus helveticus* HP	NIZO B210
Lactobacillus leichmannii	MMB 3269
Lactobacillus plantarum Qg	NIZO B253
Lactobacillus ruminis	DSM 20403
Lactobacillus salivarius subsp.*salicinius*	DSM 20554

15

Whole cell <u>in situ</u> hybridisation and enumeration of
lactobacilli in faecal samples

    For enumeration of lactobacilli in faecal samples, the
same protocol was followed as described under "Whole cell <u>in</u>

20 <u>situ</u> hybridisation and enumeration of phylogenetic clusters in
faecal samples", except that prior to dilution of the
paraformaldehyde-fixed and washed cell suspensions in 50%
ethanol, cells were resuspended in pre-lysis buffer and
incubated at 37°C for 1 hr. Cells were washed twice ((8,000 x

25 g; 5 min) in 1 ml of filtered PBS and resuspended in 1 ml of a
mixture of PBS and ethanol (1:1) and hybridized in solution as
described above.

RESULTS

Upon phylogenetic analysis, the predominant bacterial species of the human intestinal flora were distributed over 15 separate clusters. Some of these clusters constituted monophyletic genera, such as *Bifidobacterium* and *Fusobacterium*. Other were composed of a mixture of *Eubacterium*, *Clostridium*, and *Peptostreptococcus* spp., as in case of the *Clostridium coccoides* group (Larsen et al., 1993). Unique sequences for each of these separate clusters were identified, and the percentage of the total population that the probe would hybridize to was evaluated. In the case that a certain species was expected to consist a major part of the population, such as *Peptostreptococcus anaerobius*, or when a species could not be included in a cluster-probe such as *Bacterioides distasonis*, species-specific probes were developed.

The following probes were developed:

1. The *Clostridium hystolyticum* subgroup probe His5 (TTATGCGGTATTAATCT(TC)CCTT) Td = 56.5-58.4°C, has a full match with *Clostridium putrificum*, *C. butyricum*, *C. barati*, *C. carnis*, *C. botulinum*, *C. novyi*, *C. sporogenes*, *C. putrificum*, *C. tyrobytyricum*, *C. scatologenes*, *C. scatologenes*, *C. proteolyticum*, *C. limosum*, *C. sardiniensis*, *C. perfringens*, *C. beijerinckii*, *C. acebutylicum*, *C. puniceum*, *C. cellulovorans*, *C. paraputrificum*, *C. thermobutyricum*, *C. thermopalmarium*, *C. subterminale*, *C. argentinense*, *C. estertheticum*, *C. pasteurianum*, *C. histolyticum*, *Eubacterium budayi*, *E. delafieldii*, *E. moniliforme*, *E. multiforme*, *E. nitritogenes*, *E. tarantellus* and *Flexibacter canadensis*.

2. The *Clostridium lituseburense* subgroup probe Lit135 (GTTATCCGTGTGTACAGGG), Td = 59.2°C, has a full match with *Eubacterium tenue*, *Clostridium difficile*, *C bifermentans*, *C. sordellii*, *C. ghoni*, *c. lituseburense* and *C. mangenotti*.

3.   The *Peptostreptococcus* probe Pep197
(TCTTACACCGATAAACTTTG), Td = 55.4°C, has a full match with
Peptostreptococcus micros, P. tetradius, P. vaginalis and
Epilopiscium morphotye Al.

5   4.   The *Peptostreptococcus* probe Ana134
(ATGTTATCCATGTGTATAGGGC), Td = 60.2°C, has a full match with
Peptostreptococcus anaerobius and *Peptostreptococcus sp. C.*

5.   The *Fusobacterium* genus probe Fus390
(CACACAGAATTGCTGGATC), Td = 60.2°C, has a a full match with

10   *Fusobacterium simiae, F. nucleatum, F. alocis, F. russii,*
F. gonidoformans, F. necrophorum, F. varium, F. mortiferum,
F. perfoetens, Propionigenium modestum, Leptotrichia buccalis
and *Sebalcella termitidis.* The probe may also hybridize to
F. ulcerans, F. periodonticum and *F. necrogenes* since these

15   bacteria have one unknown base in the target region.

6.   The *Lactobacillus* subgroup probe Lab158
(GGTATTAGCA(TC)CTGTTTCCA), Td = 56.1-58.2°C, has a a full
match with *Lactobacillus acidophilus, L. acidophilus subsp.*
johnsonii, L. delbrueckii subsp. lactis, L. delbrueckii subsp.

20   *delbrueckii, L. brevis, L. salivarius subsp. salicinius, L.*
amylovorus, L. acetotolerans, L. oris,, L. hilgardii, L.
kefir, L. mali, L. animalis, L. murinus, L. ruminis, L.
agilis, L. sharpeae, L. gasseri, L. amylophilus, L. reuteri,
L. fermentum, L. sake, L. sanfrancisco, L. fructivorans, L.

25   *plantarum L. bifermentans, L. coryniformis, L. casei subsp.*
casei, L. casei subsp. rhamosus, Leuconostoc lactis, Lc.
mesenteroides subsp. mesenteroides, Lc. mesenteroides subsp.
cremoris, Lc. oenos, Enterococcus faecalis, E. columbae,
Periococcus pentosaceus, P. acidilactici, Streptococcus

30   *cecorum, Vagococcus fluvialis, V. salmoninarium, Weissella*
confusa and *W. kandleri.*

7. The *Streptococcus/Lactococcus* probe Str493
(GTTAGCCGTCCCTTTCTGG), Td = 61.3°C, has a full match with
Streptococcus sanguis, S. salivarius, S. bovis, S. pneumoniae,

35   *S. parasanguis, S. acidominimus, S. dysgalactiae, S.*
anginosus, S. vestibulariis, S. equinus, S. alactolytiens, S.
downei, S. sobrinus, S. rattus, S. cricetus, S.

hyointestinalis, S. agalactiae, S. suis, S. porcinus, S. canis, S. pyogenes, Lactococcus lactis subsp. *lactis, L. lactis* subsp. *cremoris, L. piscium, L. raffinolactis, L. plantarium, L. garvieae.* The probe may also be used to detect

5   *Streptococcus oralis, S. intermedius, S. macacae, S. mutans, S. anginosus, S. criae* and *S. equi* since these bacteria contain one or two unknown bases in the target region.

8.   The *Bacteroides vulgatus* assemblage probe Fra602 (GAGCCGCAAACTTTCACAA), Td = 57.0°C, has a full match with

10   *Bacteroides eggertii, B. thetaiotaomicron, B. ovatus, B. fragilis, B. uniformis* and *B. vulgatus.*

9.   The *Bacteroides distasonis* species Dis657 (CCGCCTGCCTCAAACATA), Td = 58.0°C, has a full match with *Bacteroides distasonis.*

15   10.   The Mycoplasma's walled relatives group II probe Cyl387 (CGCGGCATTGCTCGTTCA), Td = 60.2°C, has a full match with *Eubacterium cylindroides, E. tortuosum, E. dolichum, E. biforme, Clostridium innocuum* and *Streptococcus pleomorphus.*

11.   The *Atopobium* subgroup probe Ato292 (GGTCGGTCTCTCAACCC),

20   Td = 59.0°C, has a full match with *Atopobium minutum A. parvulum, A. rimae, Eubacterium lentum* and possibly als *E. fosser* (one unknown base in target region).

12.   The *Clostridium coccoides* group probe Rec484 (GCTTCTTAGTCA(AG)GTACCG), Td = 59.2°C, has a full match with

25   *Eubacterium contortum, E. formicigenerans, E. rectale, E. ventriosum, E. cellulosolvens, E. eligens, E. fissicatena, E. hadrum, E. halii, E. saburreum, E. uniforme, E. ventriosum, E. xylanophilum, Clostridium aminovalericum, C. clostridiiforme, C. nexile, C. oroticum, C. lentocellum, C. symbiosum, C.*

30   *aminophilumn, C. xylanolyticum, C.sphenoides, C. celarecrescens, Ruminococcus hansenii, Epulopiscium* sp.str. morphotypes A2 and *B. Acetitomaculum ruminis, Lachnospira pectinoschiza, L. multiparus, Roseburia cecicola* and possibly also with *Clostridium coccoides* (unknown base).

35   13. The *Ruminiococcus* probe Rum186 (TTCACACCAGACCATGCG), Td = 58.0°C, has a full match with *Ruminococcus products* and *Ruminococcus hansenii.*

14. The *Clostridium leptum* subgroup probe Lep has a full
match with *Eubacterium siraeum, E. plautii, E. desmolans,*
Clostridium sporosphaeroides, C. leptum and *C. cellulosi.*

15. The *Bifidobacterium* genus probe Bif164

5   (CATCCGGCATTACCACCC), Td = 60.2°C, has a full match with
Bifidobacterium longum, B. pseudolongum, B.suis, B. infantis,
B. indicum, B. breve, B. adolescents, B. catenulatum, B.
minimum and possibly also to *B. bifidum, B. dentium, B.*
coryneforme, B. asteroides, B. globosum, B. magnum and

10 *Gardnerella vaginalis* since these bacteria have one or more
unknown bases in the target region.

These 15 clusters were chosen as indicated in fig. 1.
Melting temperatures (Td) are theoretical values.
Hybridization will usually occur at temperatures about 1-5°C

15 below these values.
Sequences are 5'-3'.

References

1.   Amman, R.I., W. Ludwig and K.-H. Schleifer. 1995.
Phylogenetic identification of individual microbial cells
without cultivation. Microbiol.Rev.59: 143-169.

5   2.   Beimfohr, C., A. Krause, R. Amann, W. Ludwig and K.-H.
Schleifer. 1993. insitu identification of lactococci,
enterococci and sterptococci. Syst.Appl.Microbiol. 16:
450-456.

3.   Drasar, B.S. and P.A. Barrow. 1985. Intestinal
10   Microbiology. Aspects of Microbiology 10. American Society for
Microbiology, Washington, USA.

4.   Finegold, S.M., H.R. Attebery and V.L. Sutter. 1974.
Effect of diet on human fecal flora; comparison of Japanese
and American diets. Am.J.Clin.Nutr. 27: 1456-1469.

15   5.   Holdeman, L.V., E.P. Cato and W.E.C. Moore. 1997.
Anaerobe Laboratory Manual, 4th ed. Virginia Polytechnic
Institute and State University, Blacksburg, VA.

6.   Jansen, G.J., M.H.F. Wilkinson, B. Deddens and D. van der
Waaij. 1993. Characterization of human faecal flora by means
20   of an improved fluoromorphometrical method. Epidemiol.Infect.
111: 265-272.

7.   Kurat, S., K. Tamura and M. Nei. 1993. MEGA: Molecular
Evolutionary Genetics Analysis, version 1.01. The Pennsylvania
State University, University Park, PA.

25   8.   Larsen, N., G.J. Olsen, B.L. Maidak, M.J.McCaughey, R.
Overbeek, T.J. Macke, T.L. Marsh and C.R. Woese. 1933. The
ribosomal database project. Nucleic Acids Res. 21: 3021-3023.

9.   Meijer, B.C., G.J. Kootstra, D.G. Geertsma and M.H.F.
Wilkinson. 1991. Effects of ceftriaxone on faecal flora;
30   analysis by micromorphometry, Epidemiol.Infect. 106: 513-521.

10.   Porter, K.G. and Y.S. Feig. 1980. The use of DAPI for
identifying and counting aquatic microflora. Limnol.Oceanogr.
25: 943-948.

11.   Salama, M., W. Sandine and S. Giovannoni. 1991.
35   Development and application of oligonucleotide probes for
identification of *Lactococcus lactis* subsp. *cremoris*.
Appl.Environ.Microbiol. 57: 1313-1318.

12.   Sutter, V.L., D.M. Citron, M.A.C. Edelstein and S.M.
Finegold. 1985. Wadsworth anaerobic Bacteriology Manual,
4th ed. Star Publ.Corp., Belmont, CA.

13.   Van der Waaij, D., J.M. Berghuis-de Vries and J.E.C.
Lekkerkerk-van der Wees. 1971. Colonization resistance of the
digestive tract in conventional and antibiotic-treated mice.
J.Hyg. 67: 405-411.

14.   Van der Waaij, D. 1989. The ecology of the human
intestine and its consequences for overgrowth by pathogens
such as *Clostridium difficile*. Ann.Rev.Microbiol. 43: 67-87.

15.   Wilkinson, M.H.F. 1994. Shading correction and
calibration in bacterial fluorescence measurement by image
processing system. Comput.Methods Programs Biomed. 44: 61-67.

16.   Wilkinson, M.H.F., G.J. Jansen and D. van der Waaij.
1993. Very low level fluorescence detection and imaging using
a long exposure charge coupled device system, p. 221-230.
In: P.H. Bach, C.H. Reynolds, J.M. cClark. J. Mottley and P.L.
Poole (ed.), Biotechnology application of micro-injection,
microscopic imaging and fluorescence. Pergamon Press, New
York.

17.   Wilkinson, M.H.F., G.J. Jansen and D. van der Waaij.
1994. Computer processing of microscope images of bacteria;
morphometry and fluorimetry. Trends Microbiol. 2: 485-489.

CLAIMS

1. A method for determining the relative abundance of
individual species or lower phylogenetic subgroups of
microorganims in a mixed population of several microorganisms
comprising the steps of:
5   1) providing a set of labelled *in situ* hybridization
cluster oligonucleotide probes;
        2) hybridization of said probes with a sample of the
mixed population,and
        3) quantitative analysis of the number of labelled
10 microorganisms.
2. A method according to claim 1, wherein the probes are
labelled with a fluorescent material.
3. A method according to claim 1 or 2, wherein the probes
are based on 16S rRNA of target microorganisms.
15 4. A method according to anyone of the aforegoing claims,
wherein the quantitative analysis is performed by microscopic
image analysis.
5. A method according to anyone of the aforegoing claims
wherein the probes are specific for a phylogenetic cluster of
20 microorganims.
6. A method according to claim 5 wherein the phylogenetic
cluster is a genus or a species.
7. A method according to anyone of the aforegoing claims
wherein the microorganisms are bacteria.
25 8. A method according to anyone of the aforegoing claims
wherein the mixed population is the indigenous gut flora,
particular of a mammal, especially a human.
9. A method for analyzing dynamics in relative abundance of
individual microorganisms in a mixed population whereby a
30 method according to anyone of the aforegoing claims is carried
out at least twice on samples taken at a different time from
one population.
10. A method according to anyone of the aforegoing claims
wherein the sample is pretreated and/or purified.

11. A method according to anyone of the aforegoing claims wherein at least two different probes are provided with a different label.

12. A set of probes which are cluster specific and which are 5 provided with at least one label for use in a method according to anyone of the aforegoing claims.

13. A set of probes according to claim 12 being derived from bacterial 16S rRNA.

14. A set of probes according to claim 12 or 13, whereby the 10 label is a fluorescent substance.

15. A set of probes according to anyone of claims 12-14, comprising at least two different probes provided with different labels.

16. A kit of parts for determining the relative abundance of 15 individual species of microorganims in a mixed population of several microorganisms comprising a set of probes according to anyone of claims 12-15 together with suitable materials for pretreating the sample and/or suitable materials for carrying out hybridization and/or suitable materials for analysis of 20 the result of the hybridization.
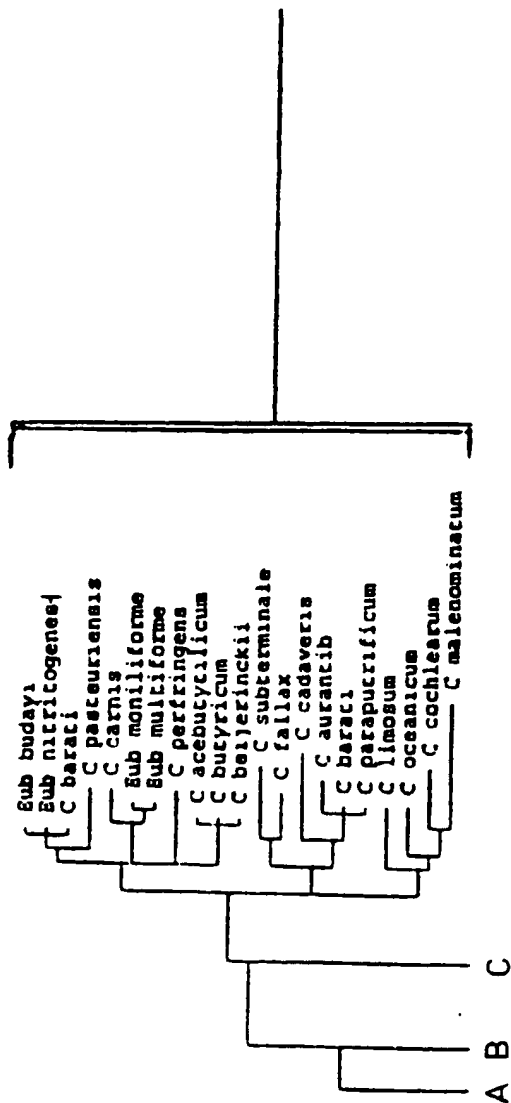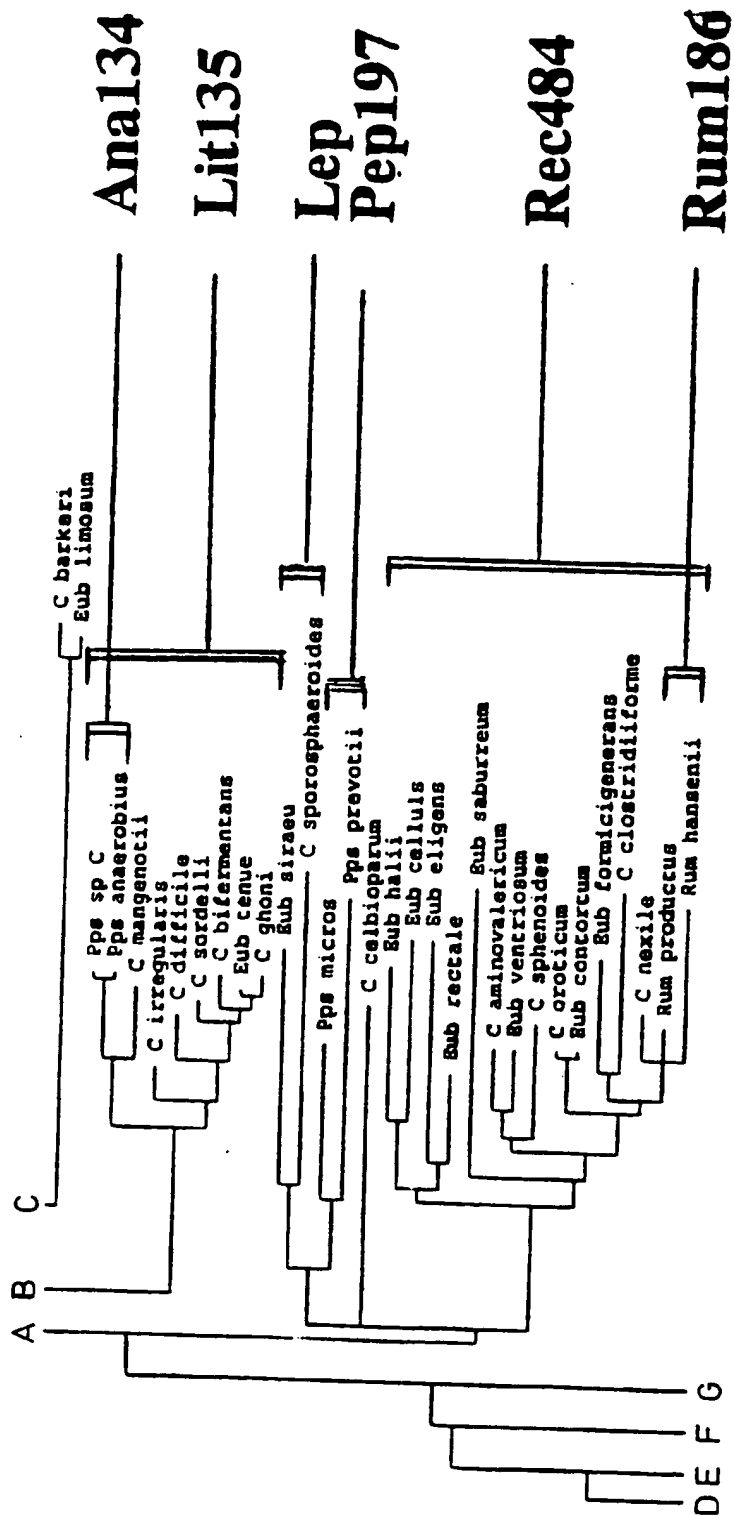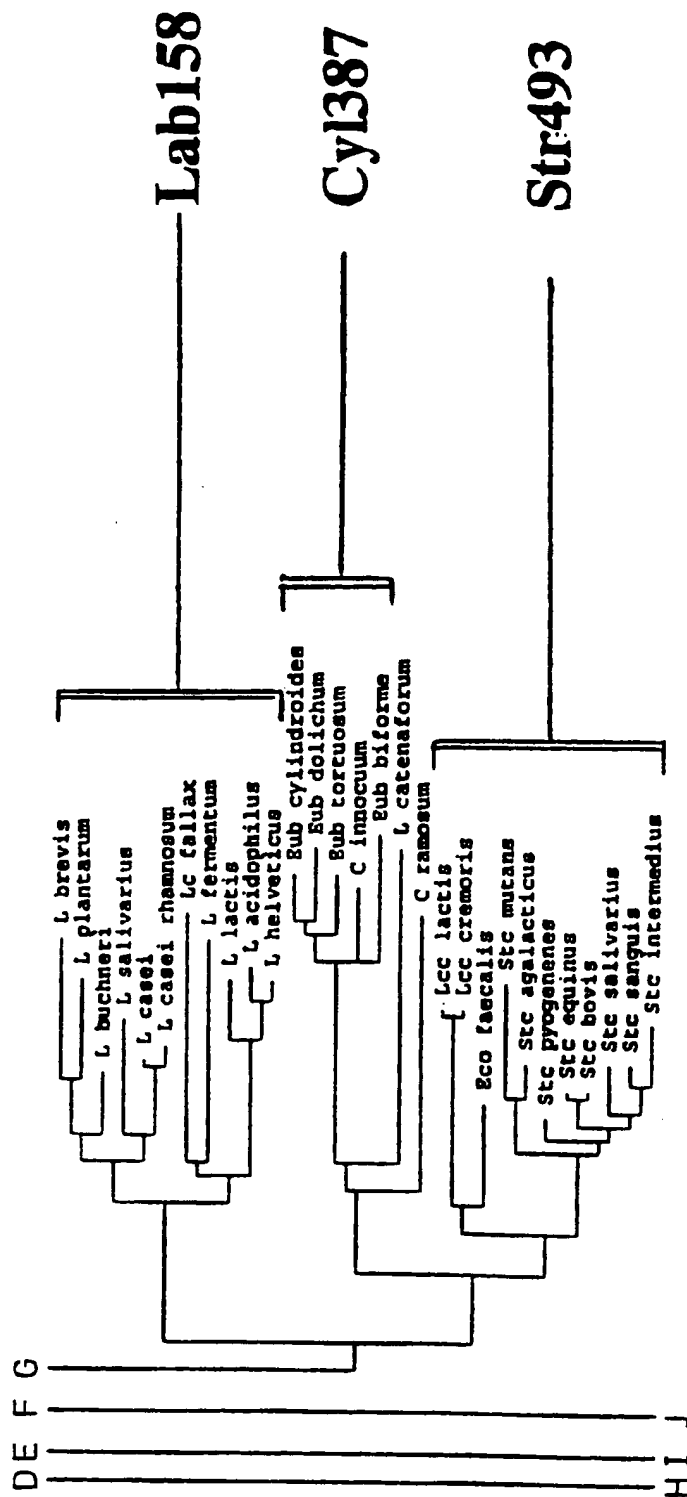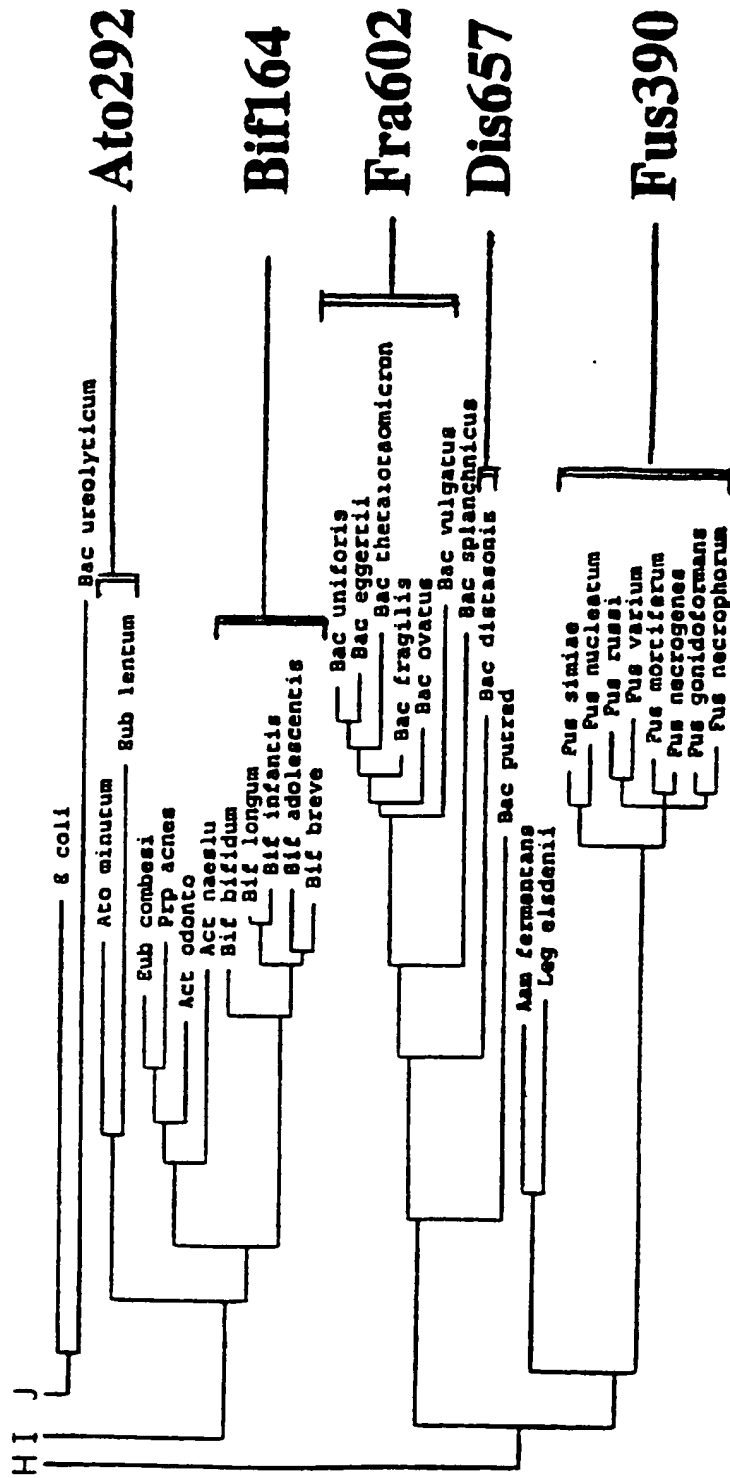
FIG. 1

FIG. 1

FIG. 1

Scale: each - is approximately equal to the distance of 0.0073185

FIG. 1

Unrooted distance tree obtained after Kimura-2 parameter analysis and neighbour joining of 200 nucleotides of the 16S rRNA sequence of bacterial species isolated from human faecal samples. Analysis was performed using MEGA software. The assignment of the 15 clusters was supported by the phylogenetic structure in bacterial small subunit data inferred by the RDP project (Larsen et al., 1993). Fluorescent oligonucleotide probes were developed for all clusters indicated.